Identification of a Serum-Induced Transcriptional Signature Associated With Type 1 Diabetes in the BioBreeding Rat

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OBJECTIVE—Inflammatory mediators associated with type 1 diabetes are dilute and difficult to measure in the periphery, necessitating development of more sensitive and informative biomarkers for studying diabetogenic mechanisms, assessing preonset risk, and monitoring therapeutic interventions.

RESEARCH DESIGN AND METHODS—We previously utilized a novel bioassay in which human type 1 diabetes sera were used to induce a disease-specific transcriptional signature in unrelated, healthy peripheral blood mononuclear cells (PBMCs). Here, we apply this strategy to investigate the inflammatory state associated with type 1 diabetes in biobreeding (BB) rats.

RESULTS—Consistent with their common susceptibility, sera of both spontaneously diabetic BB DR*lyp/lyp* and diabetes inducible BB DR+/+ rats induced transcription of cytokines, immune receptors, and signaling molecules in PBMCs of healthy donor rats compared with control sera. Like the human type 1 diabetes signature, the DR*lyp/lyp* signature, which is associated with progression to diabetes, was differentiated from that of the DR+/+ by induction of many interleukin (IL)-1–regulated genes. Supplementing cultures with an IL-1 receptor antagonist (IL-1Ra) modulated the DR*lyp/lyp* signature ($P < 10^{-6}$), while administration of IL-1Ra to DR*lyp/lyp* rats delayed onset ($P = 0.007$), and sera of treated animals did not induce the characteristic signature. Consistent with the presence of immunoregulatory cells in DR+/+ rats was induction of a signature possessing negative regulators of transcription and inflammation.

CONCLUSIONS—Paralleling our human studies, serum signatures in BB rats reflect processes associated with progression to type 1 diabetes. Furthermore, these studies support the potential utility of this approach to detect changes in the inflammatory state during therapeutic intervention. ***Diabetes*** **59:2375–2385, 2010**

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Type 1 diabetes is characterized by immune infiltration of the pancreatic islets (insulitis) and destruction of the insulin-producing β-cells. It is modeled by the biobreeding (BB) rat, in which disease is associated with insulitis, hyperglycemia, and exogenous insulin dependency (1,2). Like humans and the nonobese diabetic (NOD) mouse, the major histocompatibility complex (MHC) (insulin-dependent diabetes locus 1 [*Iddm1*]) contributes the largest genetic risk for type 1 diabetes in BB rats (3,4).

The DR*lyp/lyp* and DR+/+ congenic BB rat lines differ only by the *Iddm2* locus on chromosome 4 (5). *Iddm2* has been cloned, and the lymphopenia in DR*lyp/lyp* rats arises from a mutation in the *Gimap5* gene, which encodes a mitochondrial protein necessary for postthymic T-cell survival (6,7). The spontaneously diabetic phenotype, which occurs in 100% of DR*lyp/lyp* rats during adolescence (65.3 ± 6.3 days), is elicited through deficiency in regulatory T-cells (T_{REG} cells), as diabetes can be rescued through adoptive transfer of this population (8).

Type 1 diabetes in the nonlymphopenic BB DR+/+ rat, which possesses a wild-type *Gimap5*, is inducible through depletion of T_{REG} cells (9,10). Thus, in all BB rats, there is predisposition for type 1 diabetes that is manifest upon loss of immune regulation. This predisposition is absent in Wistar-Furth (WF) rats, which share the $RT1^u$ MHC haplotype, since depletion of T_{REG} cells does not induce disease. This susceptibility is also absent in Fischer rats, as introgression of $RT1^{u/u}$ and/or $Gimap5^{-/-}$ is insufficient for type 1 diabetes development (11).

In addition to T-cell responses, cytokines are important in diabetogenesis (12), as they are associated with β-cell destruction and disease status in both humans and spontaneous rodent models. Previously, we applied a sensitive genomics-based bioassay to investigate the presence of proinflammatory factors in human type 1 diabetes. The approach used sera of patients with recent-onset type 1 diabetes or healthy control subjects to induce transcription in unrelated healthy peripheral blood mononuclear cells (PBMCs) (13). Recent-onset type 1 diabetes sera induced a transcriptional profile that included genes related to innate immunity and genes regulated by interleukin (IL)-1. The signature was distinct from that induced by sera of healthy control subjects or long-standing type 1 diabetic patients, and analysis of a limited number of preonset samples showed that it preceded disease and the development of autoantibodies. Our findings in type 1 diabetes, and those reported for systemic-onset juvenile idiopathic arthritis (14), support that expression signatures induced by serum factors associated with different

inflammatory states are disease specific, are reflective of active disease, and are mechanistically informative.

Here, we examine the serum-induced transcriptional signatures of DR*lyp/lyp* and DR+/+ rats in an attempt to bridge the divide between invasive studies allowed in animal models and the peripheral blood sampling possible in humans. These studies identify an innate immune signature associated with progression to type 1 diabetes in DR*lyp/lyp* rats that is modulated by the addition of an IL-1 receptor antagonist (IL-1Ra) in vitro and find that administration of IL-1Ra to pre-diabetic animals modulates the inflammatory signature and delays disease onset.

RESEARCH DESIGN AND METHODS

Brown Norway (BN) and BB rats were propagated as described (5,6). Before euthanization, animals were fasted for 16 h, and those possessing blood glucose levels >130 mg/dl were disqualified from studies of pre-diabetes. Rats were anesthetized under isofluorane, and blood was collected by heart puncture. Serum was separated by centrifugation then stored at −80°C until use.

Human recombinant IL-1Ra (hIL-1Ra) (350 μg/kg/day) or vehicle (saline) was delivered to DR*lyp/lyp* rats by intraperitoneal injection. Dosage was based upon previous hIL-1Ra administration to rodents (15,16). Treatment was initiated by day 30 and continued either through day 40 or through diabetes onset. Diabetes onset was defined as the first of two consecutive blood glucose measurements >250 mg/dl. Survival was analyzed with the Kaplan-Meier method. All procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

PBMC cultures. Fresh PBMCs of healthy BN rats (~180-day-old males, to avoid variation introduced by estrous or pubertal status) were isolated by density gradient centrifugation. As described (13), transcription was induced by culturing PBMCs for 6 h at 37°C in 5% CO_2 with 20% autologous BN (self-baseline control), allogeneic BN (healthy unrelated control), DR*lyp/lyp*, or DR+/+ serum. Indicated cultures were supplemented with 1 ng/ml rat IL-1β or 1 μg/ml rat IL-1Ra. RNA was extracted using TRIzol reagent, amplified/labeled using the express kit (Affymetrix, Santa Clara, CA), and hybridized to the Affymetrix Rat Genome 230 2.0 Array. RNA from each culture was independently analyzed. Image data were quantified with Affymetrix Expression Console Software and normalized with Robust Multichip Analysis (www.bioconductor.org/) to determine signal log ratios. ANOVA was conducted and false discovery rates (FDRs) were determined using Partek Genomics Suite version 6.2. To capture the most reliable data, limit the length of gene lists, and facilitate focused pathway analyses, differentially expressed probe sets were defined as those possessing an FDR <10% and $|\log_2 \text{ratio}|$ >0.5 between the compared groups. Ontological analysis was performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (17). Hierarchical clustering was conducted with Genesis (18). Orthologous rat and human probe sets were mapped using the Affymetrix support document (HG-U133_Plus_2.na29.orthologue.csv.zip). All raw data files have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus (accession no. GSE19537).

Direct detection of inflammatory mediators. Sera of day 60 BB rats and day 180 BN rats were assayed with the BeadLyte cytokine assay kit (Millipore, Billerica, MA), per the manufacturer's protocol, and a Bio-Plex Luminex 100 XYP instrument. Concentrations were calculated with the Bio-Plex Manager 4.1 software and a five-parameter curve-fitting algorithm applied for standard curve calculations. IL-33 levels in sera and IL-1β/-α levels in conditioned culture medium were measured with Quantikine enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN), and gram-negative bacterial endotoxins (lipopolysaccarides [LPS]) were measured by the Limulus amoebocyte lysate assay (Associates of Cape Cod, Falmouth, MA).

Western blot analysis for anti–hIL-1Ra antibodies. hIL-1Ra (17 kDa) was electrophoresed through polyacrylamide gels (10–20% Tris-HCl) and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h with 5% BSA and 0.1% Tween-20 at room temperature then probed with a 1:2,000 dilution of sera from hIL-1Ra or saline-treated DR*lyp/lyp* rats overnight at 4° C. Blots were washed three times with 1 × TBS with 0.1% Tween 20 then incubated with a 1:2,000 dilution of anti-rat IgG, horseradish peroxidase–linked antibody in blocking solution for 1 h at room temperature. After three washes, the Enhanced Chemiluminescence (ECL) Plus System (Amersham, Piscataway, NJ) was used for visualization.

RESULTS

Induction of transcriptional signatures. To determine whether inflammatory mediators related to diabetogenesis could be detected through their ability to induce transcription, PBMCs of healthy, day 180 BN rats were cultured with day 60 DR*lyp/lyp* (representing the period immediately before onset), day 60 DR+/+, allogeneic BN, or autologous BN serum. To ensure induced transcription was not related to elevated blood glucose levels, only sera of normoglycemic, preonset rats were used. In preliminary studies, we observed that day 60 DR*lyp/lyp* serum, like human recent-onset sera, induced many genes regulated by IL-1. Therefore, PBMCs were also cultured with day 60 DR*lyp/lyp* serum supplemented with IL-1Ra as well as autologous BN serum supplemented with IL-1β, to respectively block or induce IL-1–mediated gene expression. Culture of BN PBMCs with DR*lyp/lyp* or DR+/+ sera regulated, within the threshold values, 1,979 and 2,904 probe sets, respectively, relative to culture with allogeneic BN sera.

Analysis of genes commonly regulated by day 60 DR+/+ and DR*lyp/lyp* sera. As reflected in the Venn diagram illustrated in Fig. 1*A*, culture of BN PBMCs with either DR*lyp/lyp* or DR+/+ sera regulated a total of 3,971 probe sets (supplement A Fig. 1*A*, available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0372/DC1). Consistent with type 1 diabetes susceptibility in both strains, the signatures share a significantly nonrandom ($P < 10^{-6}$, χ^2 test), commonly regulated intersection of 912 probe sets that represents 46.1 and 31.4% of the probe sets induced by either DR*lyp/lyp* or DR+/+ serum, respectively. Hierarchical clustering (Fig. 1*B*) shows the relatedness of the five experimental conditions for this subset. These 912 shared probe sets were annotated with DAVID, which identified significantly regulated gene ontology biological processes related to immunological activation, antigen presentation, and intracellular signaling, in particular through the nuclear factor (NF) κB pathway (Table 1 and supplement B).

NFκB is a transcriptional regulator of innate and adaptive immunity (19) that is activated through events such as binding of IL-1 to IL-1R1 or LPS to toll-like receptor (TLR) 4. These events converge at a set of I-κB kinases that phosphorylate the inhibitory I-κB proteins (NFKBIA and NFKBIB) leading to their degradation, allowing release and nuclear translocation of cytoplasmic NFκB, where it facilitates target gene transcription. Both DR*lyp/lyp* and DR+/+ sera induced *Nfkb2*, which encodes the p100 precursor that is cleaved to produce the NFκB p52 protein; the receptor-interacting serine-threonine kinase 2 (*Ripk2*), a potent activator of NFκB; as well as tumor necrosis factor (TNF) receptor superfamily member 25 (*Tnfrsf25*), which stimulates NFκB activity (20). Sera of either BB rat induced transcription of genes related to intracellular signaling, including signal transducer and activator of transcription 2 (*Stat2*) and mitogen-activated protein kinase activated protein kinase 5 (*Mapkapk5*), which is activated by MAPKs in response to exposure to proinflammatory cytokines. DR*lyp/lyp* and DR+/+ sera also induced transcription of genes encoding chemokines (*Ccl2*, *Cxcl16*) and receptors including *Tlr4*, *Tlr7*, *Ccr1*, and *Cd14*, which are important in TLR4 signaling. Other genes related to immune function included IL-1R–associated kinase 3 (*Irak3*) and intercellular adhesion molecule 1 (*Icam1*). Both DR*lyp/lyp* and DR+/+ sera induced the

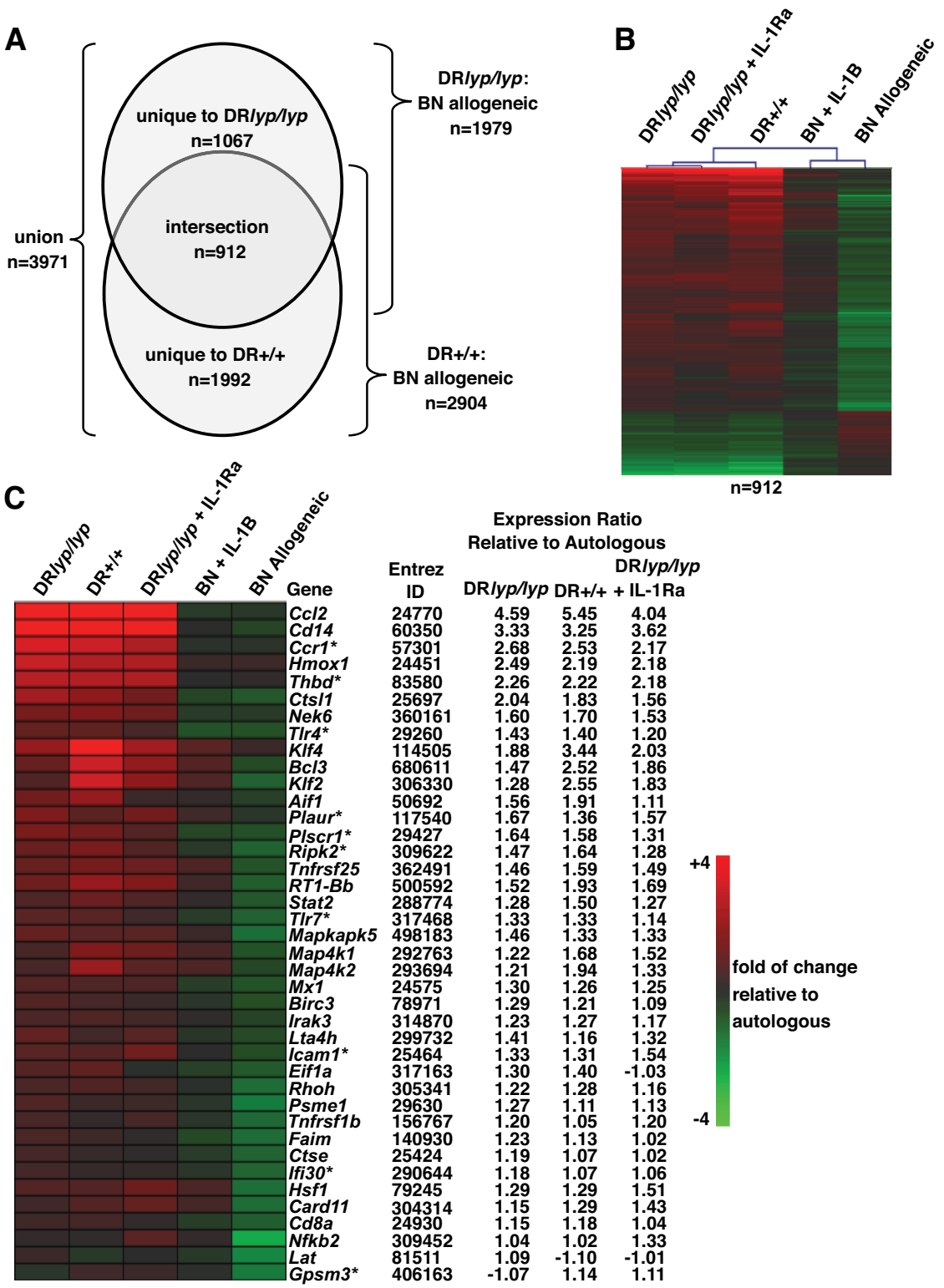


FIG. 1. Analysis of genes commonly regulated by day 60 DR+/+ and DR*lyp/lyp* sera relative to allogeneic BN sera. PBMCs of four BN rats each were cultured under six different conditions: *1*) autologous serum (n = 4 cultures), *2*) autologous serum spiked with IL-1β (1 ng/ml, n = 4 cultures), *3*) allogeneic BN serum (n = 15 cultures), *4*) a DR*lyp/lyp* serum pool (n = 4 cultures), *5*) a DR*lyp/lyp* serum pool supplemented with IL-1Ra (1 μg/ml, n = 4 cultures), and *6*) DR+/+ serum pool (n = 4 cultures). The serum pools were created from an equal contribution of six individual male rats. Gene expression was measured independently in each culture, and all data were normalized with that of the autologous induction to account for gene expression induced by placing the PBMCs into culture. *A*: A Venn diagram illustrating the relationship between the gene expression induced in the DR*lyp/lyp* vs. BN allogeneic and DR+/+ vs. BN allogeneic inductions ($|\log_2$ ratio$| > 0.5$- ± 1.4-fold; ANOVA FDR <0.10). *B*: The mean expression of the five experimental conditions were examined for relatedness by hierarchical clustering using the commonly regulated probe sets (n = 912) of the DR*lyp/lyp* vs. BN allogeneic and DR+/+ vs. BN allogeneic intersection. Note the similarity between the DR*lyp/lyp* and DR+/+ signatures and the failure of IL-1Ra to highly influence the DR*lyp/lyp* signature for this subset of genes. *C*: Well-annotated genes regulated in BN PBMCs when cultured with either DR*lyp/lyp* or DR+/+ sera related to immune activation. *Orthologues regulated by human type 1 diabetes sera (13). The scale represents the fold of change between the serum tested relative to autologous serum (−fourfold to +fourfold).

TABLE 1
Significantly regulated gene ontology biological processes

Biological process*	Count	Percentage	*P* value
Day 60 DR*lyp/lyp*:DR+/+ intersection (n = 912 probe sets)			
Antigen processing and presentation of exogenous antigen	7	0.76	2.55×10^{-4}
Immune system process	49	5.31	2.28×10^{-3}
Antigen processing and presentation	10	1.08	4.57×10^{-3}
Antigen processing and presentation of peptide antigen	8	0.87	8.62×10^{-3}
Immune response	33	3.58	1.21×10^{-2}
Regulation of transcription	87	9.44	1.89×10^{-2}
Regulation of signal transduction	33	3.58	2.68×10^{-2}
Regulation of transcription, DNA dependent	79	8.57	2.71×10^{-2}
Regulation of gene expression	91	9.87	3.54×10^{-2}
Myeloid leukocyte mediated immunity	4	0.43	3.65×10^{-2}
I-κB kinase/NFκB cascade	11	1.19	6.94×10^{-2}
Day 60 DR*lyp/lyp* (n = 1,979 probe sets)			
Antigen processing and presentation of exogenous antigen	14	0.71	6.54×10^{-9}
Antigen processing and presentation	22	1.11	4.47×10^{-6}
Antigen processing and presentation of peptide antigen	17	0.86	3.55×10^{-5}
Immune system process	95	4.80	3.50×10^{-3}
I-κB kinase/NFκB cascade	24	1.21	6.23×10^{-3}
Immune response	65	3.28	1.00×10^{-2}
Myeloid leukocyte mediated immunity	6	0.30	2.15×10^{-2}
Leukocyte migration	9	0.45	8.73×10^{-2}
Day 60 DR+/+ (n = 2,904 probe sets)			
Regulation of transcription	260	9.33	8.75×10^{-8}
Regulation of gene expression	276	9.90	2.13×10^{-7}
Regulation of transcription, DNA dependent	237	8.50	3.84×10^{-7}
Regulation of signal transduction	91	3.27	1.83×10^{-4}
Regulation of transcription from RNA polymerase II promoter	81	2.91	2.64×10^{-3}
Negative regulation of transcription, DNA dependent	38	1.36	9.95×10^{-3}
Antigen processing and presentation of exogenous antigen	8	0.29	1.21×10^{-2}
Negative regulation of transcription	49	1.76	1.34×10^{-2}
Antigen processing and presentation	17	0.61	1.40×10^{-2}
Negative regulation of gene expression, epigenetic	5	0.18	2.17×10^{-2}
I-κB kinase/NFκB cascade	25	0.90	3.33×10^{-2}
Immune system process	105	3.77	3.75×10^{-2}
Day 40 PBS-treated DR*lyp/lyp* (n = 1,526 probe sets)			
Antigen processing and presentation	27	1.73	2.63×10^{-11}
Antigen processing and presentation of peptide antigen	21	1.35	1.89×10^{-9}
Immune system process	96	6.17	1.96×10^{-6}
Immune response	67	4.30	2.31×10^{-5}
Antigen processing and presentation of exogenous antigen	9	0.58	1.92×10^{-4}
I-κB kinase/NFκB cascade	25	1.61	1.98×10^{-4}

*Genes identified in Figs. 1, 2, and 4 were analyzed for significantly regulated annotations by DAVID. If an annotation was identified in more than one of the analyses, it is listed accordingly. Complete lists of annotations are provided in supplement B. The *P* value defines the significance of the association of a particular biological process with the gene list analyzed.

transcription factor Kruppel-like factor 2 (*Klf2*), an inflammatory inhibitor that functions through the binding of coactivators necessary for optimal NFκB activity (21), and B-cell CLL/lymphoma three (*Bcl*III), an IL-10–inducible gene that impairs binding of NFκB complexes to DNA (19,22). Importantly, all 912 probe sets of the intersection exhibited directionally concordant inductions by either DR*lyp/lyp* or DR+/+ sera relative to allogeneic BN sera. Furthermore, when directly comparing expression levels between the DR*lyp/lyp* and DR+/+, only two probes sets (2 of 912, 0.2%) were differentially expressed (*Klf4* and *Map4k2*, supplement A). Overall, analysis of the intersection revealed that culturing BN PBMCs with either DR+/+ or DR*lyp/lyp* sera, compared with allogeneic BN sera, induced transcription consistent with immunological activation (Fig. 1*C*).

Independent analysis of genes regulated by DR*lyp/lyp* and DR+/+ sera. To reveal processes associated with progression to type 1 diabetes, the DR*lyp/lyp* and DR+/+ serum signatures were independently analyzed. The mean expression from each of the five experimental conditions was subjected to hierarchical clustering using the 1,979 or 2,904 probe sets respectively regulated by DR*lyp/lyp* sera or DR+/+ sera relative to the BN allogeneic induction (Figs. 2*A* and *C*). Ontological analysis of the DR*lyp/lyp* signature identified biological processes related to proinflammatory processes possessing higher numbers of associated genes with greater significance compared with the analyses of the DR*lyp/lyp*:DR+/+ intersection or the DR+/+ signature (Table 1 and supplement B). Specifically, DR*lyp/lyp* sera regulated biological processes related to antigen presentation, inflammation, and leukocyte migration. Annotated genes related to these functions included induction of *Il1b*, *Tlr2*, *Il18*, *Icam2*, proteasome subunit β type 1, (*Psmb1*), *Stat1*, and numerous MHC class II genes. Among the 1,979 probe sets regulated by DR*lyp/lyp* sera were central components of the NFκB cascade, includ-

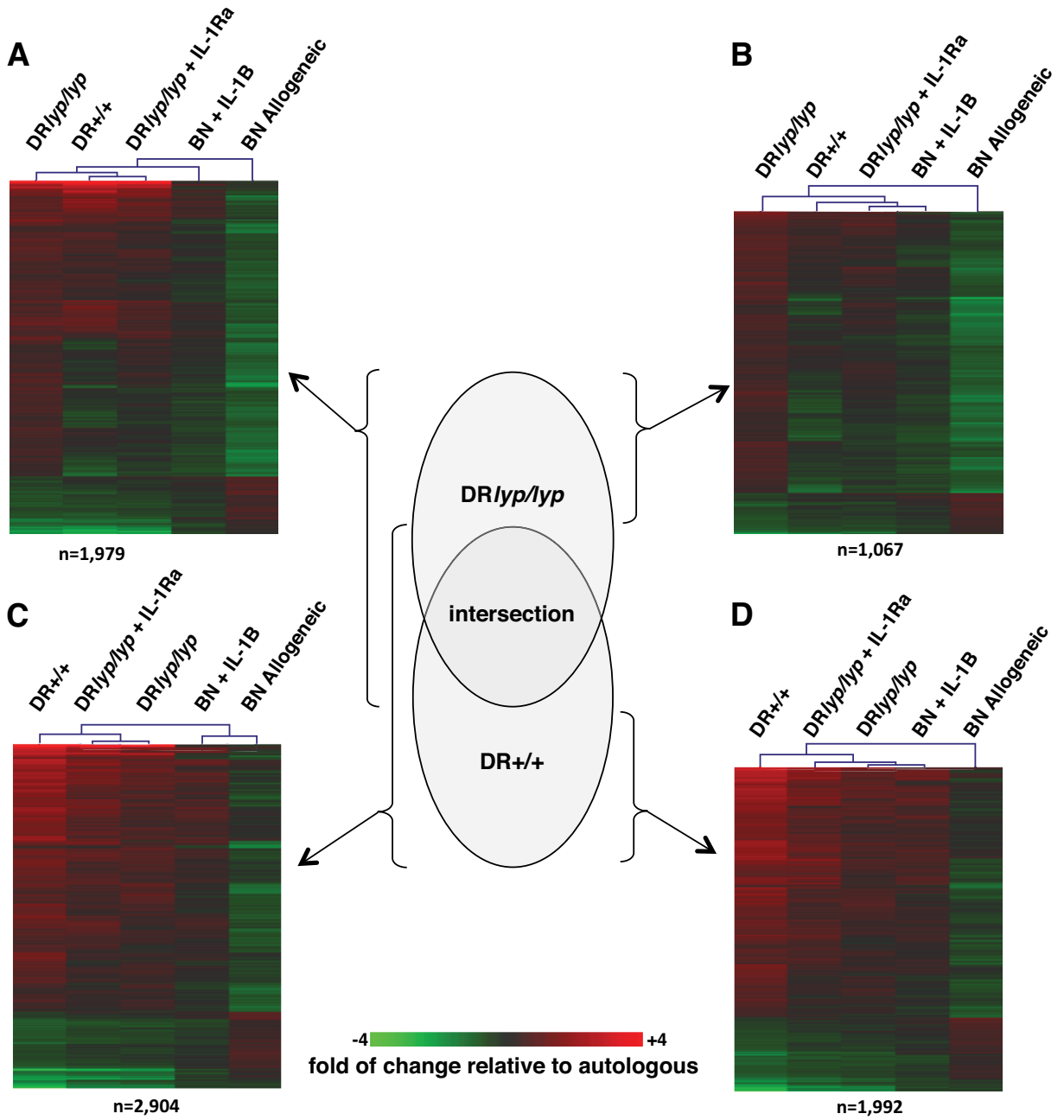
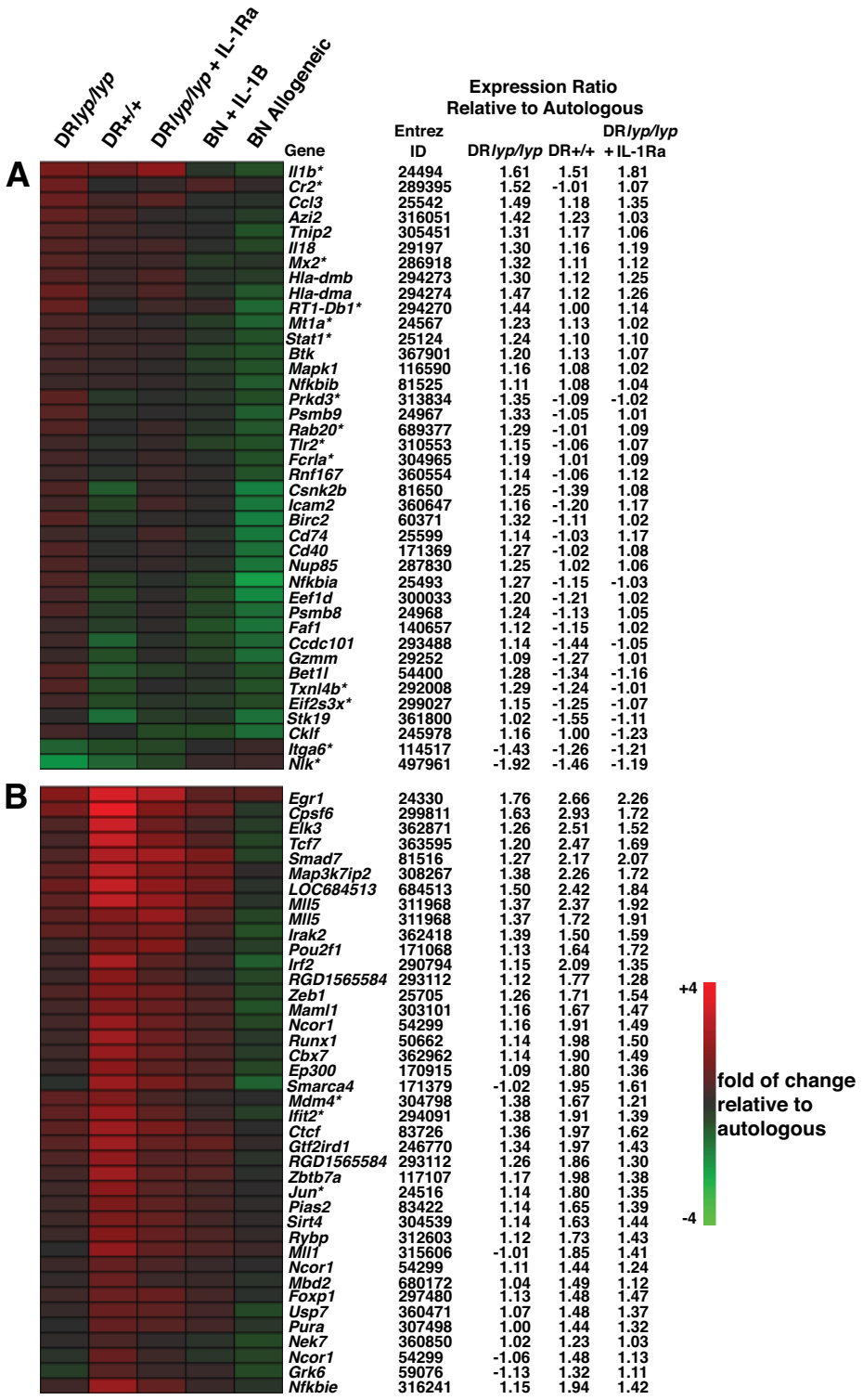


FIG. 2. Independent analysis of genes regulated by DR*lyp/lyp* and DR+/+ sera. In all cases, replicates were averaged and the relatedness of the five conditions was analyzed by hierarchical clustering. *A*: Illustrates all probe sets regulated between the DR*lyp/lyp* vs. BN allogeneic inductions including probe sets of the DR*lyp/lyp*:DR+/+ intersection (n = 1,979). *B*: Illustrates all probe sets regulated between the DR*lyp/lyp* vs. BN allogeneic inductions excluding probe sets of the DR*lyp/lyp*:DR+/+ intersection (n = 1,067). Note the distinctiveness of the DR*lyp/lyp* signature relative to the DR+/+ and the influence of IL-1Ra in impairing induction of the DR*lyp/lyp* signature in panels *A* and *B*. *C*: Illustrates all probe sets regulated between the DR+/+ vs. BN allogeneic inductions including probe sets of the DR*lyp/lyp*:DR+/+ intersection (n = 2,904). *D*: Illustrates all probe sets regulated between the DR+/+ vs. BN allogeneic inductions excluding probe sets of the DR*lyp/lyp*:DR+/+ intersection (n = 1,992). Note the similarity between DR*lyp/lyp* and DR+/+ signatures and how the addition of IL-1Ra to DR*lyp/lyp* cultures enhances expression in *C* and *D*. The scale represents fold of change between the serum tested relative to autologous serum (−fourfold to +fourfold).

ing *Nfkbia* and *Nfkbib*, as well as positive regulators of NFκB activity, including casein kinase 2 β subunit (*Csnk2b*), a potent NFκB activator that directly phosphorylates NFKBIA (23), baculoviral IAP repeat-containing 2 (*Birc2*) (20), and *Cd40* (Fig. 3*A* and supplement A).

When examining the 1,067 probe sets uniquely regulated by DR*lyp/lyp* sera (i.e., those excluding the intersection) (Fig. 2*B*), a role for IL-1 in induction of the signature became evident, as blocking IL-1R1 by adding IL-1Ra to cultures modulated the signature, yielding one more similar to that induced by DR+/+ sera. Specifically, after adding IL-1Ra to the culture, expression of 83.3% (889 of 1,067) of the DR*lyp/lyp*-specific probe sets no longer met the threshold values ($P < 10^{-6}$, χ^2 test). Among 912 genes of the intersection, only 40% (374) were influenced by the addition of IL-1Ra ($P < 10^{-6}$, χ^2 test). When examining 1,992 probe sets uniquely regulated by DR+/+ sera (Fig. 2*D*), addition of IL-1Ra to the DR*lyp/lyp* culture resulted in induction of a signature more similar to that of the DR+/+ culture in terms of fold of change. However, the overall expression levels were less influenced, in that among 1,992 genes regulated in the DR+/+ culture, only 26.2% (521 of



Gene	Entrez ID	Expression Ratio Relative to Autologous: DR*lyp/lyp*	DR+/+	DR*lyp/lyp* + IL-1Ra
*Il1b**	24494	1.61	1.51	1.81
*Cr2**	289395	1.52	-1.01	1.07
Ccl3	25542	1.49	1.18	1.35
Azi2	316051	1.42	1.23	1.03
Tnip2	305451	1.31	1.17	1.06
Il18	29197	1.30	1.16	1.19
*Mx2**	286918	1.32	1.11	1.12
Hla-dmb	294273	1.30	1.12	1.25
Hla-dma	294274	1.47	1.12	1.26
*RT1-Db1**	294270	1.44	1.00	1.14
*Mt1a**	24567	1.23	1.13	1.02
*Stat1**	25124	1.24	1.10	1.10
Btk	367901	1.20	1.13	1.07
Mapk1	116590	1.16	1.08	1.02
Nfkbib	81525	1.11	1.08	1.04
*Prkd3**	313834	1.35	-1.09	-1.02
Psmb9	24967	1.33	-1.05	1.01
*Rab20**	689377	1.29	-1.01	1.09
*Tlr2**	310553	1.15	-1.06	1.07
*Fcrla**	304965	1.19	1.01	1.09
Rnf167	360554	1.14	-1.06	1.12
Csnk2b	81650	1.25	-1.39	1.08
Icam2	360647	1.16	-1.20	1.17
Birc2	60371	1.32	-1.11	1.02
Cd74	25599	1.14	-1.03	1.17
Cd40	171369	1.27	-1.02	1.08
Nup85	287830	1.25	1.02	1.06
Nfkbia	25493	1.27	-1.15	-1.03
Eef1d	300033	1.20	-1.21	1.02
Psmb8	24968	1.24	-1.13	1.05
Faf1	140657	1.12	-1.15	1.02
Ccdc101	293488	1.14	-1.44	-1.05
Gzmm	29252	1.09	-1.27	1.01
Bet1l	54400	1.28	-1.34	-1.16
*Txnl4b**	292008	1.29	-1.24	-1.01
*Eif2s3x**	299027	1.15	-1.25	-1.07
Stk19	361800	1.02	-1.55	-1.11
Cklf	245978	1.16	1.00	-1.23
*Itga6**	114517	-1.43	-1.26	-1.21
*Nlk**	497961	-1.92	-1.46	-1.19
Egr1	24330	1.76	2.66	2.26
Cpsf6	299811	1.63	2.93	1.72
Elk3	362871	1.26	2.51	1.52
Tcf7	363595	1.20	2.47	1.69
Smad7	81516	1.27	2.17	2.07
Map3k7ip2	308267	1.38	2.26	1.72
LOC684513	684513	1.50	2.42	1.84
Mll5	311968	1.37	2.37	1.92
Mll5	311968	1.37	1.72	1.91
Irak2	362418	1.39	1.50	1.59
Pou2f1	171068	1.13	1.64	1.72
Irf2	290794	1.15	2.09	1.35
RGD1565584	293112	1.12	1.77	1.28
Zeb1	25705	1.26	1.71	1.54
Maml1	303101	1.16	1.67	1.47
Ncor1	54299	1.16	1.91	1.49
Runx1	50662	1.14	1.98	1.50
Cbx7	362962	1.14	1.90	1.49
Ep300	170915	1.09	1.80	1.36
Smarca4	171379	-1.02	1.95	1.61
*Mdm4**	304798	1.38	1.67	1.21
*Ifit2**	294091	1.38	1.91	1.39
Ctcf	83726	1.36	1.97	1.62
Gtf2ird1	246770	1.34	1.97	1.43
RGD1565584	293112	1.26	1.86	1.30
Zbtb7a	117107	1.17	1.98	1.38
*Jun**	24516	1.14	1.80	1.35
Pias2	83422	1.14	1.65	1.39
Sirt4	304539	1.14	1.63	1.44
Rybp	312603	1.12	1.73	1.43
Mll1	315606	-1.01	1.85	1.41
Ncor1	54299	1.11	1.44	1.24
Mbd2	680172	1.04	1.49	1.12
Foxp1	297480	1.13	1.48	1.47
Usp7	360471	1.07	1.48	1.37
Pura	307498	1.00	1.44	1.32
Nek7	360850	1.02	1.23	1.03
Ncor1	54299	-1.06	1.48	1.13
Grk6	59076	-1.13	1.32	1.11
Nfkbie	316241	1.15	1.94	1.42

FIG. 3. Well-annotated regulated probe sets were uniquely identified in the DR*lyp/lyp* vs. BN allogeneic induction (*A*) or the DR+/+ vs. BN allogeneic induction (*B*). *Orthologues regulated by human type 1 diabetes sera (13). The scale represents the fold of change between the serum tested relative to autologous serum (−fourfold to +fourfold).

1,992) of the probe sets in the DR*lyp/lyp* + IL-1Ra culture were regulated to meet the threshold values. These statistical findings are reflected in Figs. 2*B* and *D* and show gene expression arising through IL-1R1 signaling accounts for a large part of the difference between the DR*lyp/lyp* and DR+/+ signatures. Furthermore, addition of IL-1Ra to DR*lyp/lyp* cultures reduced gene expression annotated as proinflammatory (Fig. 2*B*) and enhanced gene expression annotated as being regulatory (Fig. 2*D*). Addition of IL-1β to PBMCs cultured with autologous BN sera induced a modest signature, more similar to that induced by that of BB rat sera than cultures possessing BN allogeneic sera (Fig. 2), regulating 209 of 3,971 probe sets regulated by DR+/+ or DR*lyp/lyp* serum relative to BN allogeneic serum.

TABLE 2
Cytokine/chemokine levels in DR*lyp/lyp*, DR+/+, and BN rats

Cytokine	Day 60 DR*lyp/lyp*	Day 60 DR+/+	Day 180 BN	Lower detection limit (pg/ml)
IL-1a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	>27
IL-1b	15.6 ± 4.4	11.7 ± 6.0	11.8 ± 8.1	>27
IL-2	20.6 ± 6.9	23.8 ± 9.7	3.9 ± 3.9	>75
IL-4	1.9 ± 1.9	5.1 ± 3.9	6.7 ± 3.3	>27
IL-5	19.6 ± 10.8	0.0 ± 0.0	0.0 ± 0.0	>10
IL-6	1.6 ± 1.3	0.0 ± 0.0	1.8 ± 1.8	>250
IL-9	224.1 ± 43.9	105.0 ± 28.5	102.9 ± 55.8	>250
IL-10	107.2 ± 30.0	58.2 ± 19.4	42.7 ± 24.2	>27
IL-13	90.4 ± 21.1*†	14.1 ± 2.2	14.1 ± 4.4	>27
IL-17	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	>10
IL-18	14.6 ± 3.0	12.4 ± 3.3	3.2 ± 1.6	>10
Granulocyte colony-stimulating factor	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	>10
Granulocyte/macrophage colony-stimulating factor	6.9 ± 5.2	0.0 ± 0.0	2.9 ± 2.9	>27
Growth-regulated oncogene alpha, chemokine CxCl1	302.5 ± 45.0	227.1 ± 29.9	199.8 ± 34.7	>27
Eotaxin	0.9 ± 0.9	0.0 ± 0.0	8.4 ± 2.8	>27
Interferon-γ	3.9 ± 2.3	1.6 ± 1.0	0.0 ± 0.0	>27
IP-10	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	>10
Leptin	1,727.4 ± 607.1	2,478.8 ± 602.3	1,438.4 ± 203.9	>10
Monocyte chemoattractant protein (CCL2)	80.0 ± 32.9	45.8 ± 21.1	87.6 ± 7.3	>75
Macrophage inflammatory protein-1 alpha	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	>10
Rantes	1,496.9 ± 416.0	6,757.0 ± 4,493.9	1,530.2 ± 176.7	>27
TNF-α	1.6 ± 0.9	0.0 ± 0.0	16.9 ± 3.1	>10
Vascular endothelial growth factor	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	>27
IL-12p70	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	>27
IL-33	8.9 ± 0.4	6.8 ± 0.1	5.6 ± 0.5	>7
LPS§	1.30 ± 0.2‡	1.34 ± 0.2‡	0.87 ± 0.1	>0.5

Data are means ± SE of six rats per group (pg/ml). Each sample was tested in duplicate using the Millipore BeadLyte cytokine assay kit, with the exception of IL-33 and LPS. *$P < 0.01$ Wilcoxon rank-sum test vs. BN; †$P < 0.01$ Wilcoxon rank-sum test DR*lyp/lyp* vs. DR+/+; ‡$P < 0.05$ Wilcoxon rank-sum test vs. BN; §conversion from endotoxin units (EU) to picograms (pg) is based upon 1 EU = 100 pg.

Overall, the ontological analysis of 2,904 probe sets regulated by DR+/+ sera revealed the greatest number of highly significant biological processes, and these annotations were primarily related to regulation of gene expression (Table 1 and supplement B). Numerous genes related to negative regulation of inflammation and NFκB signaling were regulated by DR+/+ sera, including *Nfkbie*, an inhibitor of NFκB (24); the transcriptional repressors zinc finger E-box binding homeobox 1 (*Zeb1*); zinc finger and BTB domain containing 7a (*Zbtb7a*); interferon regulatory factor 2 (*Irf2*); ETS domain-containing protein Elk-3; the inhibitor of activated STAT 2 (*Pias2*); suppressor of cytokine signaling-5 (*Socs5*); the cell cycle inhibitor; myeloid/lymphoid or mixed-lineage leukemia 5 (*Mll5*); and others (Fig. 3*B*).

While the sera of either BB substrain induced gene expression consistent with immune activation, DR*lyp/lyp* sera induced an inflammatory signature consistent with the presence of IL-1, whereas that of the DR+/+ sera can be characterized as largely immunoregulatory. Among the genes regulated by DR*lyp/lyp* and DR+/+ sera were 87 orthologues previously identified among 587 genes regulated by human type 1 diabetes sera (Figs. 1 and 3 and supplement A) (13).

Direct detection of inflammatory mediators. In an effort to account for the induced signatures, cytokine levels were measured by ELISA in the DR*lyp/lyp*, DR+/+, and BN sera used in the expression studies (Table 2). Measurable differences in IL-1α or IL-1β levels between DR*lyp/lyp*, DR+/+, or BN rat sera were not detected, raising the question whether the induced transcription measured after 6 h of culture was a primary effect of IL-1

TABLE 3
IL-1β levels in conditioned medium after PBMC culture with DR*lyp/lyp*, DR+/+, and BN sera

Duration	Day 60 DR*lyp/lyp*	Day 60 DR*lyp/lyp*+IL-1Ra	Day 60 DR+/+	Day 180 BN allogeneic	Day 180 BN autologous
0 h	2.9 ± 1.7	1.7 ± 1.2	4.4 ± 2.0	2.2 ± 1.3	2.0 ± 2.0
1 h	5.3 ± 2.3	5.3 ± 2.0	4.4 ± 1.8	0.0 ± 0.0	0.7 ± 0.8
3 h	4.7 ± 2.2	7.7 ± 6.7	4.1 ± 2.3	3.3 ± 2.9	0.8 ± 0.5
6 h	3.6 ± 1.4	5.2 ± 1.9	7.7 ± 3.5	2.7 ± 2.5	1.9 ± 0.9
12 h	7.0 ± 2.5	10.3 ± 3.8	4.6 ± 1.7	9.0 ± 4.0	6.6 ± 3.3
24 h	12.4 ± 3.3*†	10.1 ± 5.4	9.5 ± 4.1	1.2 ± 0.7	4.0 ± 1.8

Data are means ± SE of four cultures per group (pg/ml). Each culture was tested in duplicate using the IL-1β Quantikine ELISA kit (R&D Systems). In cultures possessing autologous BN sera supplemented with 1 ng/ml IL-1β, on average 816.3 ± 51.7 pg/ml was detected across the six time points. Assay sensitivity: >5 pg/ml. *$P < 0.05$ Student *t* test vs. 0 h time point. †$P < 0.05$ Student *t* test vs. day 180 allogeneic BN sera at 24 h of culture.

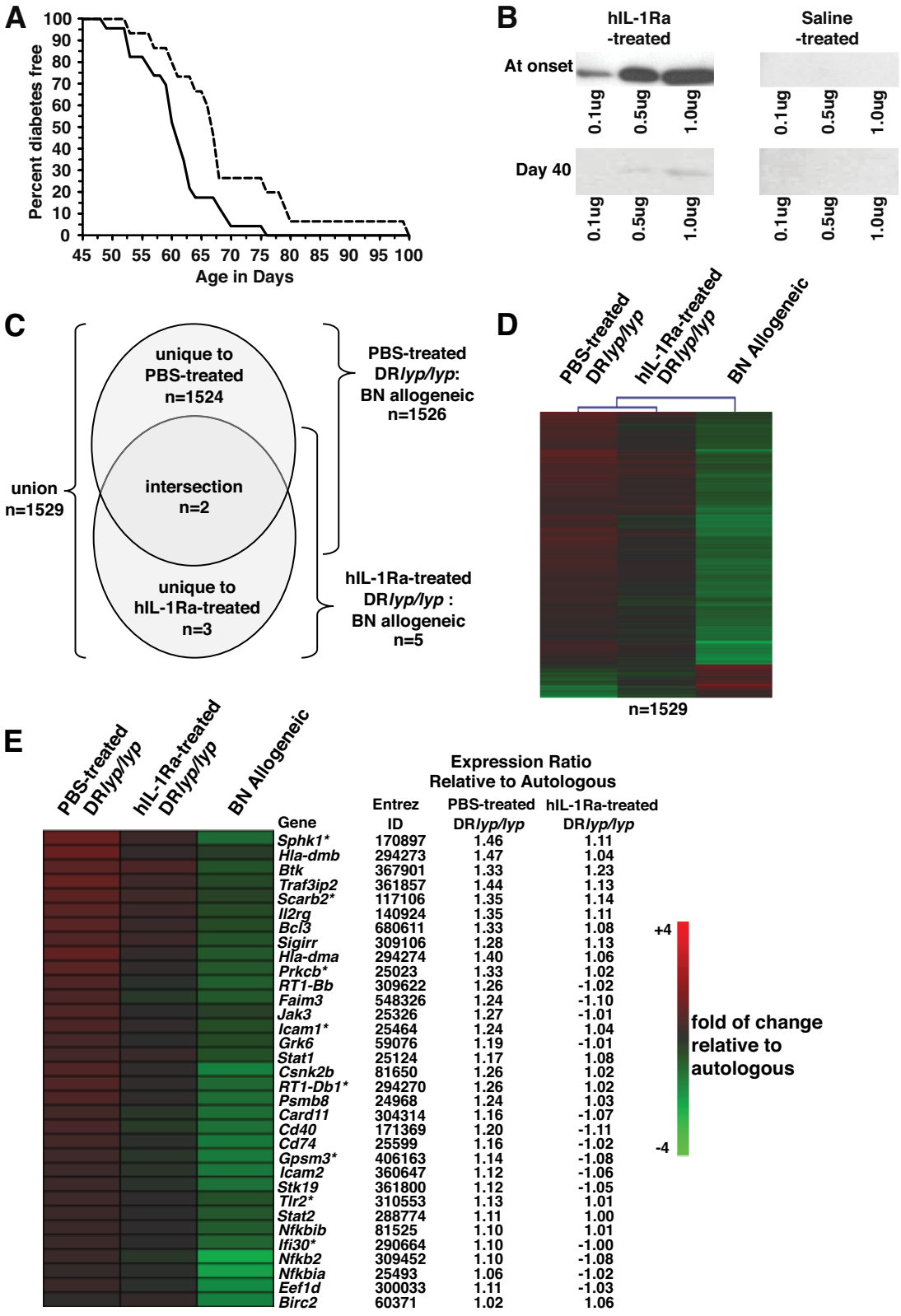


FIG. 4. Treatment of DR*lyp/lyp* rats with hIL-1Ra. *A*: Longitudinal monitoring of DR*lyp/lyp* rats treated with 350 μg/kg/day human recombinant IL-1Ra (n = 16, dashed line) or saline (n = 23, solid line). Agents were delivered intraperitoneally in saline. Treatment was initiated by day 30 (prior to insulitis). Fasting blood glucose was measured three times per week, and type 1 diabetes onset was defined as the first of two consecutive fasting blood glucose measurements >250 mg/dl. hIL-1Ra–treated rats survived 71 ± 11 days (range 53–100), while saline-treated controls survived 61 ± 6 days (53–75) (P = 0.007, log-rank test). *B*: Detection of anti–hIL-1Ra antibodies in IL-1Ra–treated DR*lyp/lyp* rats. Indicated amounts of hIL-1Ra (17 kDa) were loaded onto polyacrylamide gels, electrophoresed, and blotted. Membranes were probed with a 1:2,000 dilution of onset sera from hIL-1Ra–treated (*top left blots*), saline-treated (*top right blot*), day 40 hIL-1Ra–treated (*bottom left blot*), or saline-treated (*bottom right blot*) DR*lyp/lyp* rats. *C*: A Venn diagram illustrating the relationship between the gene expression induced between the PBS-treated DR*lyp/lyp* vs. BN allogeneic and hIL-1Ra–treated DR*lyp/lyp* vs. BN allogeneic inductions (244 $|\log_2 \text{ratio}| > 0.5$- ±1.4-fold;

on the PBMCs or a secondary effect of IL-1 produced by the PBMCs in response to other dilute mediators in DR*lyp/lyp* sera. Conditioned medium of cultures supplemented with the various sera were assayed for IL-1α and IL-1β at 0, 1, 3, 6, 12, and 24 h. While IL-1α was not detected at all, significant detectable levels of IL-1β were measured in only DR*lyp/lyp* cultures at 24 h but not at or prior to 6 h (Table 3). This supports the hypothesis that the 6 h transcriptional response is primary and is due in part to IL-1 itself, as lower IL-1β protein was not significantly detected in the DR+/+ and DR*lyp/lyp* + IL-1Ra cultures. This parallels the significant detection of *Il1b* transcript only in cultures supplemented with DR*lyp/lyp* sera. Significant differences in serum IL-10 were not observed; however, consistent with the transcriptional signatures, levels in DR+/+ and DR*lyp/lyp* serum were greater than that measured in the BN rat. IL-13 was the only significantly upregulated cytokine detected in serum of the day 60 DR*lyp/lyp* rat.

Recent studies have identified increased levels of LPS in human type 1 diabetic patients (25). Given that TLR4 and IL-1R1 signaling both are mediated through the adaptor protein MyD88 to activate NFκB, we investigated LPS serum levels. On average, significantly higher LPS levels were detected in the DR*lyp/lyp* and DR+/+ rat serum compared with that of BN rats (Table 2), consistent with the significant induction of *Il1b* transcript in cultures possessing with DR*lyp/lyp* sera even when supplemented with IL-1Ra (supplement A).

Delay of BB rat type 1 diabetes through IL-1R blockade. Given the parallels between the signatures induced by human type 1 diabetes and DR*lyp/lyp* sera, in terms of overrepresentation of transcripts regulated by IL-1 and modulation of the DR*lyp/lyp* signature upon addition of IL-1Ra to cultures, we tested the effectiveness of IL-1Ra in delaying type 1 diabetes onset in BB rats. The treatment of BB rats with rat IL-1Ra is cost prohibitive, while hIL-1Ra shares >70% amino acid homology with rat IL-1Ra and is bioactive in rat (15,26). Thus, we reasoned that if IL-1–mediated inflammation was relevant to diabetogenesis in DR*lyp/lyp* rats, treatment with hIL-1Ra should delay onset for approximately the amount of time required to mount a robust antibody response (~10–14 days). Treatment was initiated by day 30 and continued through type 1 diabetes onset. Treated rats survived 71 ± 11 days, whereas controls survived 61 ± 6 days ($P = 0.007$) (Fig. 4*A*). Significant differences in weight or growth rate were not observed between treated and control rats ($P > 0.05$). As expected, the animals possessed antibody titer to hIL-1Ra (Fig. 4*B*).

Use of transcriptional signature to monitor effect of IL-1R blockade. To determine whether hIL-1Ra treatment of DR*lyp/lyp* rats would result in modulation of the serum signature, additional rats were treated with hIL-1Ra or saline for 10 days, beginning at day 30. Serum was collected at day 40 and assayed to determine *1*) whether an inflammatory signature was present at this time prior to insulitis (27), *2*) whether day 40 and day 60 signatures were similar, and *3*) whether a day 40 signature was modulated in hIL-1Ra–treated rats. Furthermore, after only 10 days of treatment, the effect of hIL-1Ra treatment would be less likely masked by the high concentrations of neutralizing antibodies observed at onset (Fig. 4*B*, *lower panels*).

Regulated probe sets between cultures possessing serum of saline- or hIL-1Ra–treated DR*lyp/lyp* rats compared with allogeneic BN serum were identified and subjected to hierarchical clustering (Fig. 4*C* and *D*). In the PBS-treated DR*lyp/lyp* versus BN allogeneic comparison, 1,526 probe sets met these criteria, and among these, a significant percentage (638 of 1,526, 41.8%; $P < 10^{-6}$ χ^2 test) were also regulated by day 60 DR*lyp/lyp* sera. Ontological analysis again identified biological processes related to antigen presentation, inflammation, leukocyte migration, and activation of the NFκB cascade (Table 1 and supplement 2). Selected genes related to these pathways are shown (Fig. 4*E*, supplement A). Compared with BN sera, sera of day 40 DR*lyp/lyp* rats treated with hIL-1Ra regulated only five probe sets. The two probe sets of the intersection exhibited directionally concordant inductions by either saline-treated DR*lyp/lyp* or IL-1Ra–treated DR*lyp/lyp* sera relative to allogeneic BN sera. As reflected by the fold changes illustrated in Fig. 4*D*, treatment reduced induction of the proinflammatory signature observed in PBS-treated rats. Overall, these results show that like human type 1 diabetes (13), a serum-induced signature with identity to that observed at onset is detected prior to onset in the DR*lyp/lyp* rat. Moreover, this signature is modulated by treating rats with hIL-1Ra, suggesting that this approach may prove useful in monitoring the effect of therapeutic interventions in human type 1 diabetes.

DISCUSSION

Previously, we defined a transcriptional signature induced by sera of human type 1 diabetic patients (13). Here, parallel studies were conducted that defined unique signatures for diabetes-inducible DR+/+ and spontaneously diabetic DR*lyp/lyp* rats. Like human type 1 diabetes, the signature associated with disease progression in DR*lyp/lyp* rats includes many genes regulated by IL-1 and differential regulation of NFκB signaling is a key feature distinguishing the signatures induced by sera of the DR*lyp/lyp* and DR+/+ substrains.

Identity is observed between signatures induced by day 60 serum of DR*lyp/lyp* and DR+/+ rats, in particular the 912 probe sets of the DR*lyp/lyp*:DR+/+ intersection. Identity is also evident in 1,992 probe sets regulated to threshold levels by DR+/+ serum. These probe sets, annotated as immunoregulatory, are regulated by DR*lyp/lyp* serum to a lesser degree that does not meet threshold values. This suggests the presence of endogenous, albeit insufficient, immunoregulatory activity in DR*lyp/lyp* rats and may explain why relatively few (10^6) adoptively transferred DR+/+ T_{REG} cells prevent type 1 diabetes in

FDR <0.10). PBMCs of six BN rats each were cultured with a serum pool generated from six hIL-1Ra–treated DR*lyp/lyp* rats or a serum pool generated from six PBS-treated DR*lyp/lyp* rats ($n = 12$ cultures). For PBMCs of each donor BN rat ($n = 6$), a culture possessing autologous sera was prepared. Fifteen cultures possessing allogeneic BN serum were prepared. Global gene expression was measured in each culture and all data were normalized with that of the autologous induction to account for gene expression induced by placing the PBMCs into culture. *D*: Regulated probes were identified between the PBS-treated DR*lyp/lyp* vs. BN allogeneic and hIL-1Ra–treated DR*lyp/lyp* vs. BN allogeneic inductions, replicates were averaged, and the relatedness of the three conditions were examined by hierarchical clustering. *E*: Well-annotated, regulated probe sets regulated by sera of PBS-treated DR*lyp/lyp* rats vs. the BN allogeneic induction. *Orthologues regulated by human type 1 diabetes sera (13). The scale represents the fold of change between the serum tested relative to autologous serum (−fourfold to +fourfold). (A high-quality digital representation of this figure is available in the online issue.)

DR*lyp/lyp* rats (8). Conversely, from the perspective of the probe sets regulated by DR*lyp/lyp* sera, the DR*lyp/lyp* and DR+/+ signatures are distinct, with the DR*lyp/lyp* signature possessing an IL-1–driven component that is downregulated by adding IL-1Ra to the culture.

Among human type 1 diabetic patients, a significant percentage will develop autoimmune thyroid disease (28). Likewise, lymphopenic BB rats develop autoimmune thyroiditis. Since the method measures the sum of the inflammatory factors present in serum, we cannot exclude that thyroid autoimmunity has not contributed to the signature defined for DR*lyp/lyp* rats. In terms of other models, we have examined serum signatures associated with type 1 diabetes in LEW.1WR1 rats (29) and find the inflammatory states distinct but sharing partial identity (supplement C) (30).

Despite the challenges of directly measuring peripheral cytokine levels in human type 1 diabetes, studies have established that a complex cytokine milieu exists. This includes elevated IL-1 family members in patient cohorts before and after disease onset (31–35). Here, ELISA analysis was unable to detect differences in IL-1β/-α levels between DR*lyp/lyp*, DR+/+ or BN rat sera. This may be related to assay sensitivity, the limited number of rats analyzed, or presence of soluble IL-1 receptors, which impairs detection of free bioactive IL-1 and highlights the need for new, more sensitive biomarkers. Given that the amount of IL-1β (1 ng/ml) spiked into the autologous BN cultures exceeded the sensitivity of the multiplex cytokine analysis for IL-1α (>27 pg/ml) and IL-1β (>27 pg/ml), the signatures induced by DR*lyp/lyp* and DR+/+ sera (in particular genes of the intersection) are not likely an effect of IL-1 concentrations exclusively but involve the contribution of other inflammatory mediators. Binding of IL-1 and LPS to their respective receptors initiate transcriptional programs similar to those observed. We found LPS levels in BB rats nearly twice that of BN rats, consistent with reported intestinal hyperpermeability in BB rats (36), which may lead to the translocation of bacteria and/or endotoxin and a heightened systemic inflammatory state. IL-13 was elevated in DR*lyp/lyp* compared with DR+/+ and BN serum. This is consistent with elevated IgE levels and eosinophilia prior to onset in DR*lyp/lyp* rats (37), as IL-13 induces immunoglobulin isotype switching to IgE in B-cells and regulates eosinophilic inflammation.

In vitro, human and rodent pancreatic β-cells are highly susceptible to the actions of IL-1 (38), and IL-1Ra can protect β-cells from the downstream consequences of IL-1 exposure (39). While IL-1 transcript and protein are detected within immune infiltrated pancreatic islets of BB rats and NOD mice (40–42), in vivo modulation of either ligand or receptor in these models has yielded mixed results. Administration of high doses of IL-1 to diabetes-prone BB rats induced higher blood glucose concentrations before and at type 1 diabetes onset and accelerated onset (43). In contrast, administration of IL-1 to the NOD mouse delayed onset and reduced incidence (44,45). IL-1R deficiency in the NOD mouse slowed progression to type 1 diabetes but did not prevent it (46), and treatment of NOD mice with IL-1Ra inhibited recurrence of type 1 diabetes after islet transplantation (47). IL-1 has been shown to exacerbate autoimmunity by promoting expansion of effector T-cells and attenuating T_{REG} cell function (48), raising the possibility of IL-1–mediated impaired regulatory function of T_{REG} cells in DR*lyp/lyp* relative to DR+/+ rats. Treatment of DR*lyp/lyp* rats with hIL-1Ra delayed onset for the time required to mount a neutralizing humoral immune response. These results are consistent with a previous study in which hIL-1Ra delayed BB rat type 1 diabetes onset but did not affect growth or modify lymphocyte counts (16). As reported here, anti–hIL-1Ra antibodies developed, to which short-term protection (2–3 weeks) was attributed. Importantly, with delayed onset we observe modulation of the serum-induced transcriptional profile in hIL-1Ra–treated rats.

These findings support that this bioassay may not only have utility as an early mechanistically informative inflammatory biomarker of type 1 diabetes but may prove useful in monitoring changes in inflammatory state during therapeutic interventions, including those targeting IL-1 (49).

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M.K., R.G., J.B., S.P., and S.K. conducted all laboratory studies. S.J. and X.W. conducted all bioinformatic and statistical analyses. Comparative studies of serum signatures between BB and LEW.1WR1 rats were done in collaboration with J.P.M. M.K., S.P., S.K., X.W., and Å.L. reviewed/edited the manuscript. M.J.H designed/directed the study and wrote/reviewed/edited the manuscript.

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